Wiskott–Aldrich syndrome protein (WASp) is a binding partner for c-Src family protein-tyrosine kinases

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Background: Receptor-mediated signal transduction requires the assembly of multimeric complexes of signalling proteins, and a number of conserved protein domains, such as the SH2, SH3 and PH domains, are involved in mediating protein–protein interactions in such complexes. The identification of binding partners for these domains has added considerably to our understanding of signal-transduction pathways, and the purpose of this work was to identify SH3-binding proteins in haematopoietic cells.

Results: We performed affinity-chromatography experiments with a panel of GST–SH3 fusion proteins (composed of glutathione-S-transferase appended to various SH3 domains) to search for SH3-binding proteins in a human megakaryocytic cell line. Protein microsequencing identified one of the SH3-binding proteins as WASp, the protein that is defective in Wiskott–Aldrich syndrome (WAS) and isolated X-linked thrombocytopenia. WASp bound preferentially *in vitro* to SH3 domains from c-Src family kinases, and analysis of proteins expressed in insect cells using a baculovirus vector demonstrated a specific interaction between WASp and the Fyn protein-tyrosine kinase. Finally, *in vivo* experiments showed that WASp and Fyn physically associate in human haematopoietic cells.

Conclusions: Haematopoietic cells from individuals with WAS exhibit defects in cell morphology and signal transduction, including reduced proliferation and tyrosine phosphorylation in response to stimulatory factors. Members of the c-Src family of protein-tyrosine kinases, including Fyn, are involved in a range of signalling pathways – such as those regulating cytoskeletal structure – in both haematopoietic and non-haematopoietic cells. Our data suggest that binding of Fyn to WASp may be a critical event in such signalling pathways in haematopoietic cells.

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Background

Signal transduction through cell-surface receptors involves the assembly of multimeric complexes of cytoplasmic signalling proteins. Amongst the structural elements responsible for the protein–protein interactions that hold these complexes together are SH2 and SH3 domains [1]. These were first described as non-catalytic domains of members of the c-Src family of cytoplasmic protein-tyrosine kinases (PTKs), but were subsequently found in a large number of other signalling proteins [2]. Some of these — such as the p85 subunit of phosphatidylinositol 3′-kinase (PI 3-kinase), phospholipase C-γ1 (PLC-γ1), Ras GTPase-activating protein (RasGAP) and phosphotyrosine phosphatase 1C (PTP1C) — have associated enzyme activities that are important in signal transduction. Others, such as Grb2, Shc, c-Crk1 and Nck, have no associated enzyme activity but act as adaptors that couple other proteins together. Yet others, like α-spectrin, are cytoskeletal proteins.

SH2 domains bind with high affinity to selected phosphotyrosine-containing proteins [1]. Although phosphotyrosine is an essential component of SH2-binding sites, the surrounding amino acids are important in determining the affinity of binding, so particular SH2 domains show specificity for distinct phosphotyrosine-containing peptides [1]. SH3 domains bind to proline-rich sequences that form type II left-handed helical structures, and there is specificity in the binding of different SH3 domains to such sequences [1]. To investigate the function of SH3-mediated interactions, a number of groups have searched for SH3-binding proteins by screening cDNA expression libraries with labelled SH3 domains [3], by affinity chromatography followed by protein microsequencing [4], or by yeast two-hybrid screens [5]. SH3-binding proteins that have been identified include 3BP1 [3], dynamin [4], synapsin I [6], the epithelial sodium channel αENaC [7] and the guanine nucleotide-exchange factors Sos [8] and C3G [9]. Analyses of these proteins suggest that SH3 domains are involved in

diverse signalling pathways. For example, Grb2 binds via its two SH3 domains to proline-rich motifs in Sos, thereby coupling receptor PTKs to Ras [8], whereas binding of αENaC to the SH3 domain of α-spectrin seems to mediate its localization to the apical membrane of epithelial cells [7]. Using a different approach, mutational analysis of SH3 domains of c-Src family kinases has indicated that they may be involved in negative regulation of the kinase activity of these proteins [10].

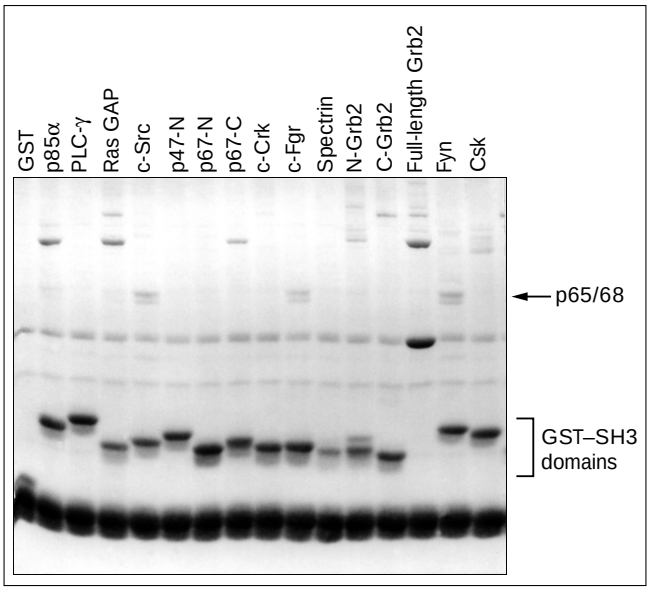
SH3-containing proteins have been implicated in a number of diverse signalling pathways in haematopoietic cells, including those triggered by receptor PTKs and by transmembrane receptors that lack kinase activity, such as B-cell and T-cell receptors and Fc receptors [11,12]. However, relatively little is known about the haematopoietic cell proteins that bind to SH3 domains in these signalling pathways. In this paper we report the use of a panel of SH3 domains in affinity-chromatography experiments to identify such proteins. Amongst the SH3-binding proteins we identified was WASp, the protein defective in the X-linked immunodeficiency disorder Wiskott–Aldrich Syndrome (WAS) [13] that has recently been linked to signalling pathways involving the Cdc42 GTPase [14,15]. WASp bound preferentially to SH3 domains from c-Src family kinases, and *in vivo* studies showed that WASp bound specifically to Fyn and, to a lesser extent, to c-Fgr. These results suggest a link between WASp and protein-tyrosine kinases in signal transduction in haematopoietic cells.

Results and discussion

Affinity purification of SH3-binding proteins

To identify SH3-binding proteins in the human megakaryocytic cell line CHRF, we incubated cell lysates with a panel of GST–SH3 fusion proteins (made up of the amino-acid sequence of glutathione-S-transferase (GST) fused to sequences encoding various SH3 domains). After extensive washing, bound proteins were analyzed by SDS–PAGE and visualized by Coomassie staining. Figure 1 shows that each GST–SH3 domain bound to a specific selection of proteins. However, the binding profile was found to be very similar for different members of the c-Src family of kinases. In particular, the most prominent of the proteins binding to the SH3 domains of c-Src, Fyn and c-Fgr was a doublet of 65/68 kDa. Binding of this doublet to SH3 domains from PI 3-kinase p85α subunit, PLC-γ and Grb2 was also just discernible (Fig. 1). These interactions were strong enough to resist washing in buffer containing a high concentration of salt and detergent. Similar analysis of other cell lysates showed that this doublet was present in a range of haematopoietic cell lines but absent from epithelial and fibroblast cell lines (data not shown). In view of the important roles of c-Src family kinases in haematopoietic cells, we were encouraged to purify and characterize p65/68.

Figure 1



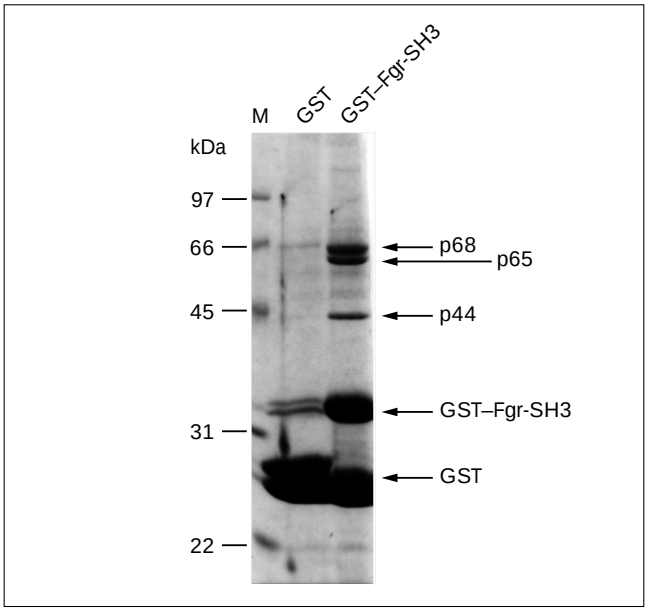
Binding of proteins from CHRF cells to a panel of GST–SH3 fusion proteins. CHRF cell lysate was incubated with glutathione–Sepharose beads coupled to GST alone or to GST fused to SH3 domains from the p85α subunit of PI3 kinase, PLC-γ, Ras GAP, c-Src, the p47 subunit of NADPH oxidase (amino-terminal SH3 domain, p47-N), the p67 subunit of NADPH oxidase (amino-terminal SH3 domain, p67-N; carboxyl-terminal SH3 domain, p67-C), c-Crk, c-Fgr, spectrin, Grb2 (amino-terminal SH3 domain, N-Grb2; carboxyl-terminal SH3 domain, C-Grb2; full-length Grb-2), Fyn and Csk. Bound proteins were eluted, analyzed by SDS–PAGE and visualized by staining with Coomassie blue. The position of the p65/68 kDa doublet and GST–SH3 fusion proteins are indicated.

The p65/68 SH3-binding protein is WASp

To identify the p65/68 SH3-binding proteins, we first performed large scale affinity purification from cell extracts of the U937 human monoblastic leukaemia cell line, because these cells were found to contain high levels of p65/68 (Fig. 2). U937 cells also contained another prominent SH3-binding protein of approximately 44 kDa (Fig. 2). Microgram quantities of p65 and p68 were excised from the SDS–PAGE gel and digested with lysoendopeptidase C. Generated peptides were resolved by high-pressure liquid chromatography and their amino-acid sequences determined. As shown in Figure 3a, some of the peptides were common to both p65 and p68, suggesting that the two proteins were closely related.

Searches of the protein databases showed that all of the peptides generated from p68 were identical to equivalent sequences in WASp (Fig. 3b), the recently identified product of the *WAS* gene at Xp11.22-p11.23, which is defective in people with WAS and isolated X-linked thrombocytopenia [16–21]. However, only six of the eleven peptides generated from p65 matched WASp sequences. The remaining peptides showed no significant homology

Figure 2



Binding of p65/68 from U937 cell lysates to a GST fusion protein containing the c-Fgr SH3 domain (GST–Fgr-SH3). U937 cell lysates were in incubated with glutathione–Sepharose beads coupled to GST alone or to GST–Fgr-SH3. Bound proteins were eluted, analyzed by SDS–PAGE and visualized by staining with Coomassie blue. The positions of the p65/68 kDa doublet, an SH3-binding protein of approximately 44 kDa, the GST–Fgr-SH3 fusion protein and GST are indicated by arrows. M, molecular weight markers.

to WASp or to any proteins in the database. It is possible that the affinity-purified 65 kDa band contained more than one protein, one of which was WASp. Alternatively, p65 could be an isoform of WASp, perhaps encoded by an alternatively spliced mRNA.

Analysis of the *in vitro* association between WASp and a panel of SH3 domains by immunoblotting

To further characterize the interaction between WASp and SH3 domains, we raised a polyclonal rabbit antiserum (SK3) against a carboxy-terminal peptide corresponding to amino acids 487–501 of WASp [13]. This antiserum was affinity purified using a carboxy-terminal peptide–Actigel matrix. In immunoblotting experiments, affinity-purified SK3 antibodies specifically recognized a U937 cell protein of approximately 68 kDa, which bound to GST–Fgr-SH3 but not to GST alone (Fig. 4). SK3 antibodies did not recognize p65 (Fig. 4), suggesting either that p65 and p68 differ at their carboxyl termini, or that differences elsewhere in the proteins result in different conformations at their carboxyl termini. Further confirmation of the specificity of affinity-purified SK3 antibodies came from their ability to bind to WASp expressed using a baculovirus expression system (see below). SK3 antibodies were not able to immunoprecipitate WASp from cell lysates.

We used affinity-purified SK3 antibodies in immunoblotting experiments to re-examine the binding of WASp to the panel of GST–SH3 fusion proteins. Silver staining (Fig. 4a) and immunoblotting analysis (Fig. 4b) clearly

Figure 3

(a)

p68

F77	TPGAPESSALQPPPQSSEGLVGALMHVMQ
F76	HVSHVGWDPQN
F80	GRSGPLPPVPLGIAPPPPT
F37	ADIGAPSGFK

p65

F37	ADIGAPSGFK
F43	EHXGAVXFVK
F71	RNQRQSGDRRQLPP(P)GT(S)A
F69	LIYDFIEDQGFLEAVRQEMR
F76	TPGAPESSALQPPPQ
F76	HVSHVG
F77	PFELGLEFPNLPYYID
F68	GAGAGGPLP
F35	TVTNDRSAPILDK
F46	LARNESRSGSNR(R)E
F48	TEQAGXNALLSDISK

(b)

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  1 MSGGPMGGRP GGRGAPAVQQ NIPSTLLQDH ENQRLFEMLG RKCLTLATAV
 51 VQLYLALPPG AEHWTKEHCG AVCFVKDNPQ KSYFIRLYGL QAGRLLWEQE
                    EHXG AVXFVK
101 LYSQLVYSTP TPFFHTFAGD DCQAGLNFAD EDEAQAFRAL VQEKIQKRNQ
                                                    RNQ
151 RQSGDRRQLP PPPTPANEER RGGLPPLPLH PGGDQGGPPV GPLSLGLATV
    RQSGDRRQLP PPGTSA
201 DIQNPDITSS RYRGLPAPGP SPADKKRSGK KKISKADIGA PSGFKHVSHV
                                    ADIGA PSGFKHVSHV
251 GWDPQNGFDV NNLDPDLRSL FSRAGISEAQ LTDAETSKLI YDFIEDQGGL
    GWDPQN                                  LI YDFIEDQGFL
301 EAVRQEMRRQ EPLPPPPPPS RGGNQLPRPP IVGGNKGRSG PLPPVPLGIA
    EAVRQEMR                               GRSG PLPPVPLGIA
351 PPPPTPRGPP PPGRGGPPPP PPPATGRSGP LPPPPPGAGG PPMPPPPPPP
    PPPPT
401 PPPPSSGNGP APPPLPPALV PAGGPGPGGG RGALLDQIRQ GIQLNKTPGA
                                                    TPGA
451 PESSALQPPP QSSEGLVGAL MHVMQKRSRA IHSSDEGEDQ AGDEDEDDEW
    PESSALQPPP QSSEGLVGAL MHVMQ
501 DD
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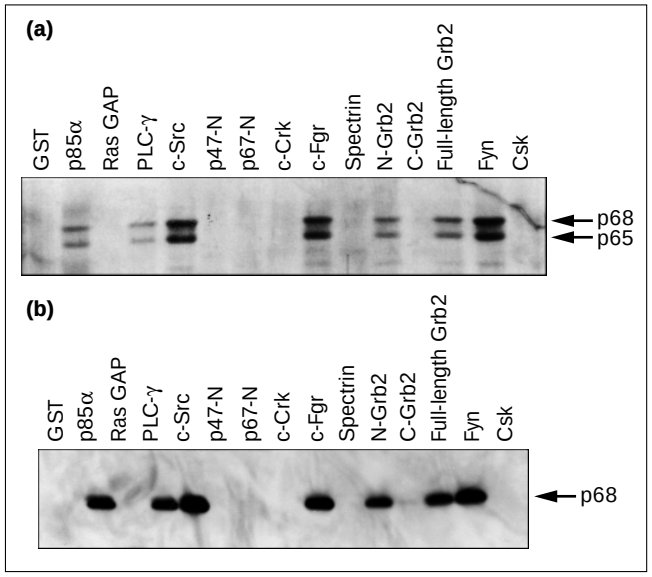
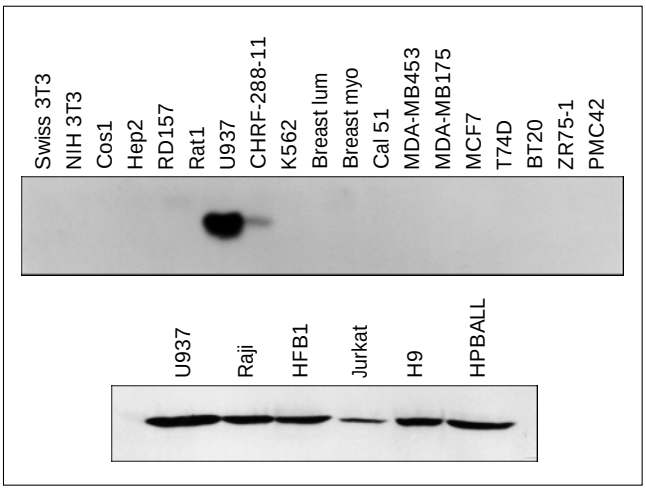
(a) Amino-acid sequences of peptides (single-letter code) obtained from lysoendopeptidase C digest of p65 and p68. Sequences shown in red are found in WASp. The identities of the amino acids shown in parenthesis are uncertain; 'X' denotes unknown amino acids. (b) Alignment of p65 and p68 peptides (in red) with the amino-acid sequence of WASp (in black) [13].

Figure 4



Binding of proteins from U937 cells to a panel of GST–SH3 fusion proteins. U937 cell lysate was incubated with glutathione–Sepharose beads coupled to GST alone or to GST fused to SH3 domains from the p85α subunit of PI 3-kinase, Ras GAP, PLC-γ, c-Src, the p47 subunit of NADPH oxidase (amino-terminal SH3 domain, p47-N), the p67 subunit of NADPH oxidase (amino-terminal SH3 domain, p67-N; carboxyl-terminal SH3 domain, p67-C), c-Crk, c-Fgr, spectrin, Grb2 (amino-terminal SH3 domain, N-Grb2; carboxyl-terminal SH3 domain; C-Grb2; full-length Grb-2), Fyn and Csk. Bound proteins were eluted, subjected to SDS–PAGE and visualized by silver staining (a) or by immunoblotting using SK3 antibodies directed against WASp (b). The positions of p65 and p68 proteins are indicated by the arrows.

Figure 5



Cell distribution of WASp. Total cell lysates of a range of cell lines were subjected to SDS–PAGE and immunoblotted using the anti-WASp antibody SK3. Upper panel: Swiss 3T3; NIH 3T3; Cos1; Hep2; RD157; Rat1; U937; CHRF-288-11 (human megakaryocytic leukaemia); K562 (human erythroid leukaemia); breast lum (primary human breast luminal epithelial cells); breast myo (primary human breast myoepithelial cells); Cal51. MDA-MB453, MDA-MB175, MCF7, T47D, BT20, ZR75-1 and PMC42 are human breast carcinoma cell lines. Lower panel: U937; Raji (human B-lymphoma); HFB1 (human plasmacytoma); Jurkat (human T-cell leukaemia); H9 (human T-cell leukaemia); HPB-ALL (human T-cell leukaemia). SK3 antibodies also recognized rodent WASp in rodent haematopoietic cells (data not shown). The visible band is p68.

confirmed our original observation that WASp binds preferentially to SH3 domains in c-Src, Fyn and c-Fgr, and, to a lesser extent, to the SH3 domains of PLC-γ, Grb2 and the p85α subunit of PI 3-kinase. In the case of Grb2, WASp bound to the amino-terminal SH3 domain rather than the carboxy-terminal SH3 domain, and binding was retained in full-length Grb2 protein.

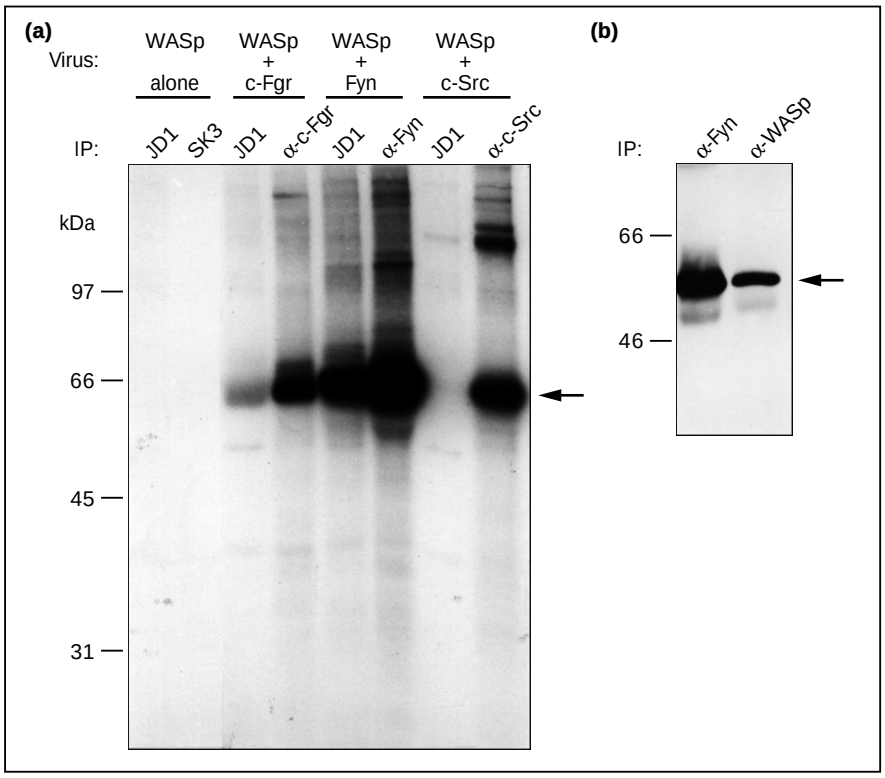
Cell distribution of WASp

Total cell lysates from various cell lines were resolved by SDS–PAGE, immunoblotted and incubated with anti-WASp (SK3) antibodies. WASp was expressed in all haematopoietic cell lines tested, including U937, Raji, HFB1, Jurkat, H9, HPBALL, CHRF and HL60, but was not expressed in any of the epithelial, myoblast and fibroblast cell lines that were examined (Fig. 5 and data not shown). These results suggest that WASp expression is restricted to cells of haematopoietic lineages. This parallels earlier studies of the distribution of *WAS* mRNA [13].

WASp associates specifically with Fyn in insect cells

To determine whether the ability of WASp to bind to isolated SH3 domains *in vitro* reflected an ability to bind to full-length SH3-containing proteins *in vivo*, we first cloned the full-length WASp coding region into a baculovirus transfer vector. The recombinant virus directed high-level expression of WASp in Sf9 insect cells, as judged by its immunoreactivity with anti-WASp (SK3) antibodies and its ability to bind to the GST–Fgr-SH3 fusion protein. The electrophoretic mobility of the expressed WASp corresponded to that of the 68 kDa protein detected in haematopoietic cells by immunoblotting (data not shown). We also constructed a baculovirus encoding human c-Fgr (data not shown) and used baculoviruses encoding human c-Src or human Fyn. We co-expressed WASp with c-Src, Fyn or c-Fgr by co-infecting Sf9 cells with the appropriate baculoviruses. Three days after infection, cells were lysed and immunoprecipitations performed using an immunoprecipitating anti-WASP antiserum (JD1) [15]. To determine whether c-Src, Fyn or c-Fgr were present in the immunoprecipitates, we first performed immune complex kinase assays and looked for autophosphorylated c-Src, Fyn or c-Fgr by SDS–PAGE. As shown in Figure 6a, anti-WASp immunoprecipitates from cells co-infected with WASp and Fyn viruses contained protein kinase activity. The major co-precipitated phosphoprotein co-migrated with Fyn that was immunoprecipitated from the co-infected cells by an anti-Fyn antiserum (Fig. 6a). Immunoblotting

Figure 6



In vivo association between WASp and SH3-containing proteins. (a) Sf9 cells were infected with a baculovirus encoding WASp, alone or in combination with baculoviruses encoding c-Fgr, Fyn or c-Src. Cell lysates were incubated with immunoprecipitating antibodies directed against WASp (JD1), c-Fgr (α-c-Fgr), Fyn (α-Fyn) or c-Src (α-c-Src), or with non-immunoprecipitating anti-WASp antibodies (SK3). Immunoprecipitates were analyzed by immune complex kinase assay followed by SDS–PAGE, and phosphorylated proteins were visualized by autoradiography. The major co-precipitating phosphoproteins are indicated by the arrow. (b) Sf9 cells were co-infected with baculoviruses encoding WASp and Fyn. Immunoprecipitates were prepared from cell lysates using anti-Fyn or anti-WASp (JD1) antisera, and were subjected to SDS–PAGE and immunoblotted using the anti-Fyn antiserum. The position of Fyn is indicated by the arrow.

of anti-WASp immunoprecipitates using the anti-Fyn antiserum confirmed that Fyn co-precipitated with WASp (Fig. 6b). Anti-WASp immunoprecipitates from cells co-infected with viruses expressing WASp and c-Fgr also contained protein kinase activity, but this was significantly weaker than that seen in WASp–Fyn immunoprecipitates (Fig. 6a). Anti-WASp immunoprecipitates from cells co-infected with viruses expressing WASp and c-Src did not contain any detectable kinase activity (Fig. 6a). We were unable to detect c-Fgr or c-Src in anti-WASp immunoprecipitates by immunoblotting (data not shown). These results show that WASP can associate *in vivo* with Fyn and, much less strongly, with c-Fgr, but not with c-Src.

WASp and Fyn are physically associated in U937 cells

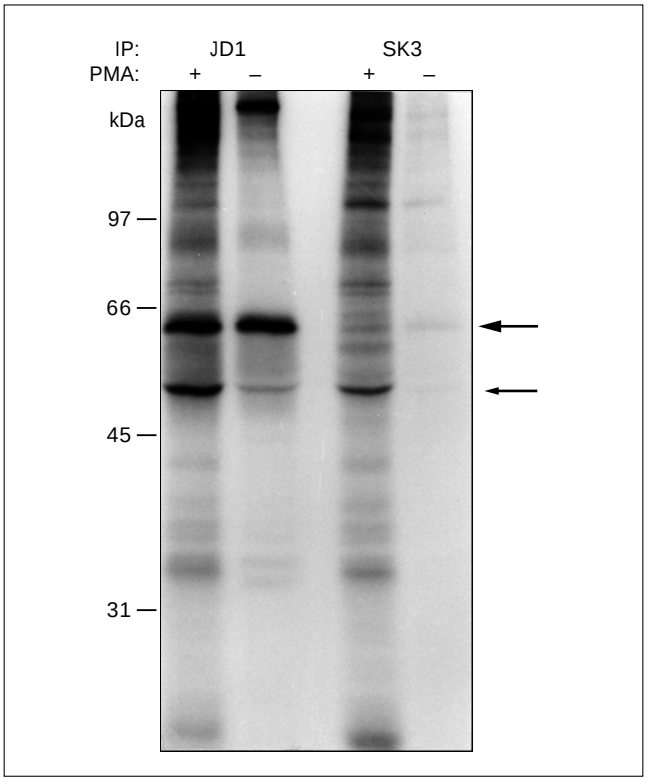
Anti-WASp immunoprecipitates from U937 cell lysates also contained kinase activity, and the major co-precipitating phosphoprotein co-migrated with Fyn upon SDS–PAGE (Fig. 7). Immunoblotting with a monoclonal anti-Fyn antibody confirmed that anti-WASp immunoprecipitates contained Fyn (Fig. 8), demonstrating that WASp and Fyn are normally associated in U937 cells.

Conclusions

WAS is characterized by a broad range of clinical abnormalities, including thrombocytopenia, eczema, and increased susceptibility to infection. Several different immunological abnormalities have been described and there is an increased risk of lymphoid malignancy [22]. The gene that is mutated in WAS has recently been identified [13], and our results indicate that there is a specific interaction *in vivo* between the protein product of this gene (WASp) and the cytoplasmic protein-tyrosine kinase Fyn, which is mediated by the SH3 domain of Fyn. This is the first report of an association between WASp and a tyrosine kinase *in vivo*, although WASp has previously been shown to bind to the adaptor protein Nck via its SH3 domains [23] and to Cdc42 by a mechanism that does not involve SH3 domains [14,15]. The biochemical consequences of these associations remain to be determined, although our preliminary data suggest that WASp may be phosphorylated, and the kinase activity of Fyn stimulated, in WASp–Fyn complexes (data not shown). Nevertheless, the fact that WASp interacts with Cdc42 and Fyn suggests that it may have an important role in a number of different signalling pathways in haematopoietic cells, which is consistent with the broad range of abnormalities seen in WAS.

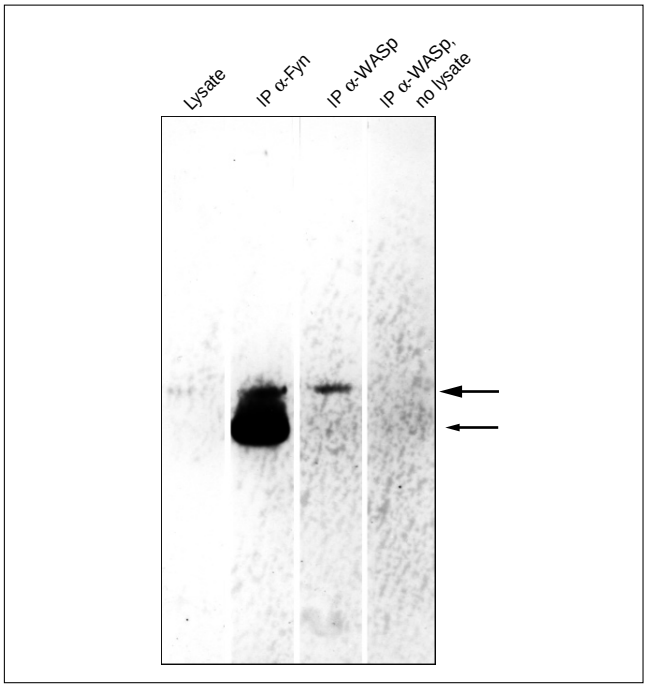
WASp contains a number of proline-rich motifs that could behave as SH3-binding sites; these motifs are mostly located in the carboxyl-terminal half of the protein [13]. One or more of these could be responsible for the binding of WASp to Fyn. Patients with WAS or X-linked thrombocytopenia have missense, nonsense or frameshift mutations

Figure 7



Co-precipitation of WASp and a protein kinase from U937 cells. Lysates of U937 cells, untreated or treated with phorbol 12-myristyl 13-acetate (PMA), were incubated with immunoprecipitating JD1 anti-WASp antibodies or non-immunoprecipitating SK3 anti-WASp antibodies. Immunoprecipitates were analyzed by immune complex kinase assay and phosphorylated proteins were visualized by autoradiography. The large arrow indicates a phosphoprotein that co-migrates with Fyn and is found specifically in immunoprecipitates containing WASp. The small arrow indicates a smaller phosphoprotein that is enriched in PMA-treated U937 cells and is present in negative-control SK3 immunoprecipitates that do not contain WASp.

Figure 8



Co-precipitation of WASp and Fyn from U937 cells. Total cell lysate from U937 cells (lysate) and U937 cell lysate immunoprecipitated with a mouse anti-Fyn monoclonal antibody (IP α-Fyn) or with JD1 rabbit anti-WASp antiserum (IP α-WASp) were subjected to SDS–PAGE and immunoblotted using the mouse anti-Fyn monoclonal antibody. The large arrow indicates that Fyn protein is present in U937 cells, in anti-Fyn immunoprecipitates and in anti-WASp immunoprecipitates. The secondary antibody used for immunoblotting recognizes the heavy chain of the mouse anti-Fyn monoclonal antibody, which migrates slightly faster than Fyn, as indicated by the small arrow. However, the secondary antibody does not cross-react with the rabbit anti-WASp antiserum, as indicated by a control in which cell lysis buffer was immunoprecipitated with the anti-WASp antiserum (IP α-WASp, no lysate).

that are found throughout the *WAS* gene, but are concentrated in the first two exons [16,17]. None of the mutations characterized to date lies within the proline-rich motifs that are candidates for SH3 binding. However, if SH3 binding can be mediated by more than one of these motifs, it is possible that a mutation in any one of them would not have any functional consequences.

A number of studies have shown that haematopoietic cells from WAS patients have morphological abnormalities, suggesting that WASp may involved in maintaining or regulating cytoskeletal structure. For example, platelets from WAS patients are abnormal in size and shape, and thrombocytopenia seems to result from to their accelerated destruction [24]; T-cells from WAS patients have a decrease in the size and density of microvillus surface projections [25]. Cdc42 is involved in regulating cytoskeletal structure, and its interaction with WASp may be important in this context [14,15]. However, c-Src family PTKs, including Fyn, also seem to have important roles in regulating cytoskeletal changes in both haematopoietic [26] and non-haematopoietic [27,28] cells, and it is possible that the interaction between WASp and c-Src family PTKs is involved in signalling pathways that regulate the cytoskeleton in haematopoietic cells.

Other work suggests that WASp has a role in signalling pathways that regulate proliferative responses of lymphocytes, and our data raise the possibility that this involves an interaction with Fyn. For example, cross-linking of the B-cell receptor on B cells from WAS patients fails to induce cell proliferation or tyrosine phosphorylation of cellular proteins, including PLC-γ1 [29], and there is good evidence that Fyn is involved in signalling mediated by the B-cell receptor [12]. Likewise, T-cells from WAS patients fail to proliferate in an oxidative mitogenesis assay [30], and Fyn has an important role in signal transduction mediated

by the T-cell receptor [11], which involves tyrosine phosphorylation of PLC-γ1 [31–33]. It is therefore possible that interaction between WASp and Fyn is important for signalling downstream of the B- and T-cell receptors, as well as for signalling events in other haematopoietic lineages.

Materials and methods

Cell lines

U937 cells were maintained in RPMI 1640 medium supplemented with 5 % fetal bovine serum. To induce differentiation towards the monocytic lineage, exponentially growing U937 cells were sub-cultured at a density of 5×10^5 cells ml^{-1} and treated with PMA (Sigma) at a final concentration of 10 ng ml^{-1} for 24 h. CHRF-288-11 cells were maintained in RPMI 1640 medium supplemented with 20 % heat-inactivated horse serum.

Preparation of cell and tissue lysates

Cells were washed in ice-cold phosphate-buffered saline and lysed in 10 mM Tris–HCl, (pH 7.5), 1 % (v/v) Triton X-100, 150 mM NaCl, 5 mM EDTA, 50 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 5 mM benzamidine, 1 mM $NaVO_4$ and 100 kallikrein inhibitor units ml^{-1} of aprotinin (lysis buffer). Protein concentrations were measured using the Bradford assay.

GST–SH3 fusion protein affinity chromatography

We previously reported the construction and expression of a panel of GST–SH3 fusion proteins [4]. Affinity matrices were prepared by immobilising GST alone or GST–SH3 fusion proteins on glutathione–Sepharose beads (Pharmacia), as described [34]. Freshly prepared cell or tissue lysates were placed on ice for 20 min, clarified by centrifugation at 15 000 rpm for 20 min and incubated with the affinity matrices for 2 h at 4 °C. After extensive washing, bound proteins were released by boiling in SDS sample buffer and analysed by SDS–PAGE. SH3-binding proteins were visualized by silver or Coomassie staining.

Purification and sequencing of SH3-binding proteins

Glutathione–Sepharose beads pre-loaded with approximately 50 μg of GST–Fgr-SH3 fusion protein were incubated with lysate from 2×10^8 U937 cells for 2 h at 4 °C. After extensive washing in lysis buffer, bound proteins were fractionated by SDS–PAGE. Proteins were visualized by Coomassie staining and the two bands in the 65/68 kDa doublet were excised separately and digested with lysylpeptidase C. Peptides were separated by reverse-phase high-pressure liquid chromatography (Hewlett Packard 1090M) and sequenced using a modified Applied Biosystems 477A Sequencer [35].

Antibodies

Rabbits were immunized with a carboxy-terminal peptide, corresponding to amino-acids 487–501 of WASp [13], coupled to activated keyhole limpet hemocyanin. Immunoreactive serum SK3 was selected for further use and was affinity purified on a carboxy-terminal peptide–Actigel affinity matrix. A second anti-WASp antiserum (JD1), was raised against a GST–WASP fusion protein containing amino acids 48–321 of WASp [15]. A mouse monoclonal anti-Fyn antibody was obtained from Santa-Cruz Biotechnology.

Immunoprecipitation and immune complex kinase assays

Freshly prepared cell lysates were incubated for 2 h at 4 °C with the specific primary antibody, and then for 1 h at 4 °C with protein A–Sepharose beads. In some experiments, cell lysates were incubated with primary antibody that had been covalently cross-linked to protein A–Sepharose beads. Immune complexes were collected by centrifugation, washed extensively with lysis buffer and boiled in SDS sample buffer for prior to analysis by SDS–PAGE.

Protein-tyrosine kinases were detected by immune complex kinase assay. After immunoprecipitation and extensive washing in lysis buffer, the beads were washed once in kinase buffer (50 mM Hepes (pH 7.5), 150 mM NaCl, 2 mM $MnCl_2$, 12 mM $MgCl_2$, 100 mM $NaVO_4$, 10 % v/v glycerol and 1 mM DTT) and 10 μCi γ-[^{32}P]ATP was added. Samples were incubated at room temperature for 15 min and then analyzed by SDS–PAGE followed by autoradiography.

Immunoblotting

Proteins were transferred from SDS–PAGE gels by electroblotting on to polyvinylidene difluoride (PVDF) membrane (Millipore, Massachusetts). Membranes were blocked by incubation for 1 h at room temperature in blocking buffer, consisting of phosphate-buffered saline (PBS), 0.05 % (v/v) Tween and 4 % (w/v) dried milk. Membranes were then incubated with primary antibody (affinity purified SK3 anti-WASp at a dilution of 1/1000 in blocking buffer) for 2 h at room temperature, washed in blocking buffer, and incubated with horseradish peroxidase-conjugated anti-rabbit Ig (Amersham) for 1 h at room temperature. Finally, membranes were washed twice in blocking buffer and once in PBS containing 0.1% (v/v) Tween, and immunoreactive proteins were detected by enhanced chemiluminescence (Amersham).

Generation of baculoviruses.

Full-length human *WAS* cDNA [13] and human *c-fgr* cDNA [36] were cloned into the pAcSG2 and pAc36C baculovirus transfer vectors (PharMingen), respectively. Sf9 insect cells were transfected with the transfer vectors and viral genomic DNA (Baculo Gold) using lipofectin. Recombinant viruses were amplified and insect cells infected as described [4].

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